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Photosystem II associated carbonic anhydrase activity in higher plants is situated in core complex

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Abstract The thylakoid membrane containing photosystem II (PSII membranes) from pea and wheat leaves catalyzed the reaction of CO_2 hydration with low rate, which increased after their incubation either with Triton X-100, up to Triton/chlorophyll ratio 1:1, or 1 M $CaCl_2$. The presence of the inhibitor of CAs, *p*-aminomethylbenzensulfonamide (mafenide), at the start line in the course of electrophoresis of PSII membranes solubilized by *n*-dodecyl-β-maltoside (DM) decreased the amount of PSII core complex in the gel. The elution of PSII core complex from the column with immobilized mafenide occurred only either by mafenide or another inhibitor of CAs, ethoxyzolamide. The above results led to a conclusion that membrane-bound CA activity associated with PSII is situated in the core complex.

Keywords: Carbonic anhydrase; C_3-plant; Chloroplast; Photosystem II; Core complex

1. Introduction

Now, it is possible to assert that thylakoid membranes of chloroplasts of higher plants possess carbonic anhydrase (CA) activity distinct in characteristics from the activity of a soluble CA [1,2]. The literature data show that substantial part of the thylakoid CA activity is structurally associated with photosystem II (PSII) [3–5]. Elucidation of the role of CA activity in PSII requires ascertainment of its exact localization in the photosystem. Previously, we revealed that the pea thylakoid membrane fragments enriched with PSII demonstrated a noticeable dehydrase CA activity only after the treatments either with detergent or salt, while this activity was high in the core complex of PSII [6,7]. The presence of CA activity was also found in PSII core complexes from *Chlamydomonas reinhardtii* [8]. Using the affinity electrophoresis and affinity chromatography of *n*-dodecyl-β-maltoside (DM) extract of PSII membrane isolated from wheat, we got in the present work new convincing data, which permitted to locate PSII CA activity in the core complex.

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Abbreviations: CA, carbonic anhydrase; Chl, chlorophyll; CP43 and CP47, chlorophyll-binding proteins with molecular weights 43 and 47 kDa, respectively; DM, *n*-dodecyl-β-maltoside; EZ, ethoxyzolamide; LHC, light-harvesting complex; mafenide, *p*-aminomethylbenzenesulfonamide; PSII, photosystem II

2. Materials and methods

Thylakoid membrane fragments enriched with PSII (PSII membranes) from wheat were prepared mainly as in [9]. The isolated chloroplasts were incubated 2 min with Triton X-100 at detergent/chlorophyll (Chl) ratio of 25 (w/w) in the medium 'A' (50 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$ and 400 mM sucrose) in the ice bath under stirring. The mixture was then centrifuged at $5000 \times g$ for 10 min and a supernatant was centrifuged at $28\,000 \times g$ for 30 min. The pellet was suspended up to Chl concentration of 0.5 mg/ml in 'A' medium containing 0.1% Triton X-100 and centrifuged at $28\,000 \times g$ for 30 min. The pellet was washed from Triton by its suspension in the 'A' medium followed by the same centrifugation. The final pellet contained PSII membranes with high capacity to reduce 2,6-dichlorophenolindophenol from water. The PSII membranes from pea were prepared according to [10]. The membrane preparations supplied with 10% glycerol were frozen in liquid nitrogen and stored at −80 °C.

Suspension of pigment–protein complexes in DM-extract was prepared as follows. DM was added into suspension of PSII membranes (1 mg Chl/ml) in the medium containing 50 mM MES-KOH (pH 6.0), 1 M betaine, 5 mM $MgCl_2$, and 10 mM NaCl to a ratio DM/Chl of 15:1 (w/w); this mixture after incubation at stirring on ice during 30 min in the dark was 10 times diluted with 50 mM MES-KOH (pH 6.0) and then centrifuged at $28\,000 \times g$ for 1 h. Sucrose and $MgCl_2$ were added into supernatant at concentrations of 0.4 M and 5 mM, respectively, and were centrifuged at $28\,000 \times g$ for 1 h. This procedure removed a considerable part of light-harvesting complex (LHC) and was used to enrich the preparation with the core complex [11]. The final supernatant was concentrated and essentially released from the detergent by three times centrifugal filtration using Centriprep-30 tubes (Amicon).

Affinity electrophoresis was carried out in 7.5% PAAG in the tubes at 1 mA per tube for 7–8 h at 4 °C in the dark. 0.5 M betaine was included into the gels before polymerization in order to stabilize the pigment–protein complexes. The start surface of gels was covered either with 1-cm layer of agarose (control gels) or 1-cm layer of agarose with covalently bonded *p*-aminomethylbenzenesulfonamide (mafenide) (agarose/mafenide) (Sigma). Deriphate-160 was added to electrode buffer as in [12]. The gels after electrophoresis were scanned photometrically at 430 nm using spectrophotometer equipped with a special device. The positions of Chl–protein complexes in gels were identified by estimating molecular mass using standard markers in parallel gel as well as, after cutting out the bands from gels, both by measuring low-temperature fluorescence spectra [13] and by performing, in some cases, the denaturing electrophoresis.

Affinity chromatography was conducted by loading DM-extract (10 ml with 8.6 mg of Chl) on the column (10×5) filled up with agarose/mafenide. The fractions were analyzed by measuring the absorbance at 280 nm as well as at 405 nm using chromatograph FPLC (Pharmacia). The rate of washing chosen in the separate experiments was 0.1 ml/min.

Soluble proteins (used in the experiments presented in the inset of Fig. 1) were purified from pea leaves after grinding with pestle in the

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presence of broken glass in the cooled mortar in the buffer containing 0.1 M Tris–H_2SO_4 (pH 8.1), 5 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and Polyclar AT (2% of leaves weight). Homogenate was centrifuged at $150 \times g \times 1$ min, then the supernatant was centrifuged at $13\,000 \times g \times 30$ min and a new supernatant – at $70\,000 \times g \times 30$ min. The supernatant after last centrifugation was used. Protein content was determined after trichloroacetic acid precipitation according to [14].

CA activity as CO_2 hydration was measured at 2 °C in 13.6 mM veronal buffer (pH 8.4). Water, being saturated with CO_2 at 0 °C during 1 h, was added to the reaction mixture up to 36% of the final volume, and the rate of a pH decrease from 8.3 to 7.8 was recorded. CA activity was calculated as the difference between the rates in the presence and in the absence of biological preparation in the medium. The titration with HCl was conducted in both cases to take into account the difference in the buffer capacities. CA activity was expressed either on Chl or protein basis.

Chlorophyll was determined according to [15] in ethanolic extracts.

3. Results

Fig. 1 shows that hydrase CA-activity of a suspension of PSII membranes increased at the addition of Triton X-100 up to detergent/Chl ratio (w/w) of 1 and then decreased under a further rise in this ratio. Such dependence was observed with all preparations studied and the increase in CA activity could be even fivefold if the initial activity of PSII membranes was low. Triton X-100 did not affect the activity of soluble CA from pea leaves (inset in Fig. 1). To find out whether an increase in the CA activity of the suspension of PSII membranes in the presence of detergent is conditioned by an increase of the membrane activity, and whether the CA activity appears in the solution, the activities of the supernatant and the pellet after centrifugation were measured (Table 1). With both PSII membrane preparations, from pea and wheat, the CA-activities of supernatants after sedimentation of membranes treated either with Triton X-100 or $CaCl_2$ were not detected. Lu and Stemler [4] observed CA activity in the supernatants after the similar salt treatment of PSII membranes, after desalting of these supernatants. The absence of CA activity in the supernatants, that were not desalted (Table 1), showed that an increase of CA activity in Fig. 1 was the result of an increase in the activity of the membranes per se. The gain in CA activity of PSII membranes pellets (Table 1) after both treatments was indicative of strong binding of the CA activity to PSII. The drop of CA-activity in Fig. 1 is in accord with a decrease in stimulation of the CA activity of pea PSII membranes at high Triton concentration [7]. Since Triton inhibited the CA activity of the isolated core complex, the latter effect was explained by a suppression of deeply buried PSII CA activity [7].

To localize more precisely the position of CA activity in PSII, we studied which component(s) of PSII membrane would be bound to the specific inhibitor of CA, mafenide, bonded to

Table 1
CA activities of PSII membranes from pea and wheat leaves and of aqueous phases of corresponding suspensions after incubation of the membranes either with Triton X-100 or $CaCl_2$

Additions in incubation medium[a]	Source of PSII membranes	CA activity, μmol H^+ (mg Chl min)$^{-1}$	
		Supernatant	Pellet
–	Wheat	n.f.[b]	40
	Pea	n.f.	65
Triton X-100 at Triton/Chl of 1 (w/w)	Wheat	n.f.	150
	Pea	n.f.	87
1 M $CaCl_2$	Wheat	n.f.	188
	Pea	n.f.	189

[a] PSII membranes were incubated 20 min at 0–2 °C in the medium containing 20 mM MES-KOH (pH 6.5), 15 mM NaCl, 10 mM $MgCl_2$, 0.4 M sucrose, without and with indicated additions, and then were centrifuged at $28\,000 \times g$ (wheat) or at $13\,000 \times g$ (pea) for 40 min; pellets were suspended in the same medium without any additions.
[b] n.f., not found.

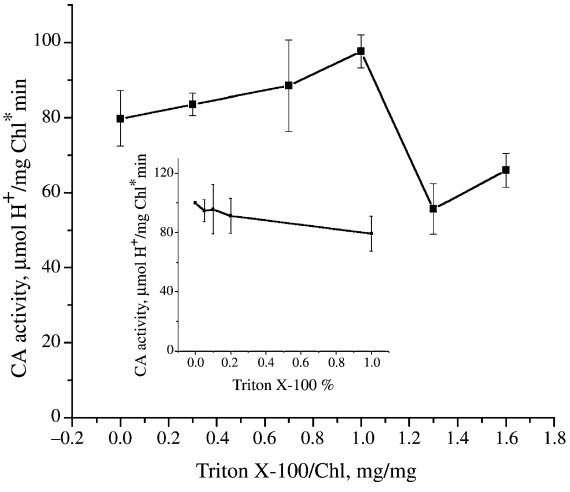

Fig. 1. The effects of Triton X-100 on CA activities of suspensions of PSII membranes (main panel) and soluble proteins from pea leaves (inset). The membranes and soluble proteins were incubated at 0 °C for 20 min at various Triton concentrations as indicated, and then the activities of the mixtures were measured.

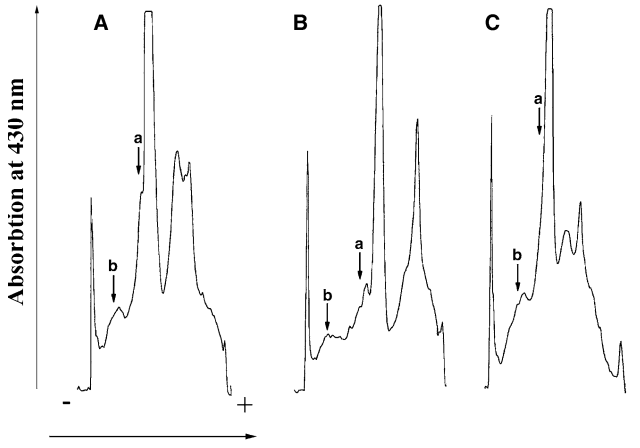

Fig. 2. The scan profiles of PAA gels after affinity electrophoresis of dodecylmaltoside extracts from PSII membranes isolated from wheat. The amount of loaded extract was equalized according to the Chl content, 200 μg on each gel. The start lines of the gels were covered either by 1-cm layer of free agarose (A) or 1-cm layer of agarose with bonded mafenide (B and C). In C, the solution of bovine CA in 50 mM Tris–HCl (pH 7.2) plus 0.2 M sucrose passed through agarose/mafenide before loading DM-extract. The letters 'a' and 'b' near the arrows indicate the position of the monomer and the dimer of the core complex of PSII, respectively.

neutral matrix. Fig. 2 shows the results of electrophoresis of a PSII membrane extract that passed either through a free agarose or agarose with bonded mafenide. Several green bands were seen in the bulk of the gel, and the most dense band corresponded to dimer of LHC, which intentionally was not completely removed from the DM-extract by sedimentation and centrifugation (see Section 2) in order to have the reference point to follow the behavior of all pigment–protein complexes. The bands that had a higher electrophoretic mobility than LHC had contained chiefly CP43 and CP47; these proteins could not be separated under these conditions. The 'a' and 'b' bands were the monomer and dimer of core complex, that was proved by denatured electrophoresis of these fractions. It may be seen from Fig. 2 that when agarose with bonded mafenide was at the start (part 'B'), these bands were noticeably less than when a free agarose was (part 'A'). When bovine CA was passed through the agarose/mafenide before DM-extract of PSII membranes (part 'C'), the amount of the core complex in the gel became close to its amount in the gel when DM-extract passed through a free agarose (part 'A').

After electrophoresis was done, the presence of Chl (green layer) at the start zone was observed only in the case of the presence of agarose/mafenide. Chlorophyll was absent in the case of a free agarose and a weak greenish layer was there when agarose/mafenide was supplied with bovine CA. This observation led to another approach to identify a carrier of CA activity in PSII. Fig. 3 shows chromatography of DM-extract on the column with immobilized mafenide. It is seen that the outlet of extract's components depended on the composition of the elution buffer. The initial drop in absorption at 405 nm in this figure indicates termination of the outlet of free pigments and LHC (that was checked using spectral analysis and denaturing electrophoresis of this fraction). The insertion of 0.3 M NaCl into a basic buffer to elute the hydrophilic proteins led to an insignificant additional outlet of the pigments. To wash the column from the pigment-protein complexes bound possibly to agarose and/or mafenide hydrophobically, we added 0.1% Triton X-100 while NaCl concentration in elution buffer was diminished to 15 mM. The fraction, which was removed by this combination, contained mainly LHC and a few of other Chl–protein complexes connected non-specifically with agarose. After this washing, the green bands were preserved in the column with agarose/mafenide, while the control column filled with agarose without mafenide lost all Chl.

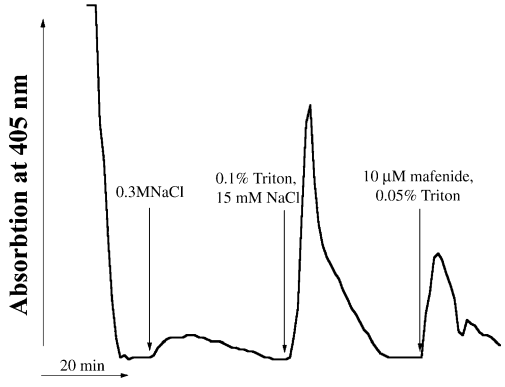

Fig. 3. Elution of Chl-containing fractions from a chromatography column loaded with dodecylmaltoside extract of PSII membranes isolated from wheat under varying the components of the elution buffer. See text on a qualitative composition of the fractions.

Finally, the affinity column was washed with the buffer in which either 10 μM mafenide (Fig. 3) or 1 μM ethoxyzolamide (EZ) (not shown) in the presence of 0.05 % Triton X-100 was added. I_{50} values for inhibition of the CA activity of the thylakoid membranes, which were washed out from contamination with soluble CA, were found to be 2×10^{-4} and 4×10^{-7} M for mafenide and EZ, respectively (Rudenko, in preparation). The pigment–protein complexes, which were eluted now, displayed spectral and electrophoretic characteristics of a dimer and a monomer of the PSII core complex and possessed activity for reduction of DPIP from diphenylcarbazide (not shown).

4. Discussion

An increase in hydrase CA activity of the PSII membrane after treatment either with Triton X-100 or $CaCl_2$ (Table 1) is similar to an increase in their dehydrase CA activity [7]. CO_2 being added to measure the former activity is able to traverse lipid phase easier than HCO_3 being added to measure the latter activity. Thus, the low value of dehydrase CA activity of PSII membrane was not only due to the presence of hydrophobic barrier for HCO_3^-, as supposed earlier [7]. If these low activities did not result from structural changes due to isolation procedure, and if $CaCl_2$ and Triton X-100 only 'unclothed' the activity (i.e., did not stimulate it themselves), then we may conclude that the reaction center site of the carrier of the CA activity in PSII is deeply buried inside the photosystem. This would have a physiological meaning, ensuring the prompt removal of protons released during water oxidation. The latter process can be a part of the system preventing photoinhibition, as was proposed previously [8].

The Mn-stabilizing protein 33 kD was considered as a possible carrier of the CA activity [4]. However, this protein passes into solution in the course of $CaCl_2$ treatment of PSII membranes [16]. At the same time, under our assay conditions, CA activity was not observed in the solutions after this treatment (Table 1).

The data presented in Figs. 2 and 3 provide convincing evidence that the carrier of CA activity is a part of the core complex. The fact that the supply of agarose/mafenide with bovine CA (Fig. 2C) did decrease binding of the core complex with mafenide (it should be noted that the same result was obtained when bovine CA was added into the DM-extract before loading on to PAA gel) implied that CA activity of the core complex was a consequence of the presence of a component possessing this activity. Mafenide can bind CAs owing to low dissociation constant of its complex with metal situated in an active center of these enzymes. Thus, the core complex component possessing CA activity must have an active metal-containing center, the structure of which resembles the structure of the known CAs. However, the metal is not obligatory to be zinc. Zinc addition was found to inhibit CA activity of PSII [3,7]. The substitution of zinc for cobalt did not lead to a significant decrease in the activity of the bovine CA [17]. Moreover, iron- and cobalt-substituted forms of CA from *Methanosarcina thermophila* exhibited greater CO_2 hydration rates than the zinc enzyme [18]. Thus, it is not improbable that manganese within the water-oxidizing complex can be involved in the CA activity.

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References

[1] Moskvin, O.V., Ignatova, L.K., Ovchinnikova, V.I. and Ivanov, B.N. (1995) Biochemistry (Moscow) 60, 859–864.
[2] Ignatova, L.K., Moskvin, O.V., Romanova, A.K. and Ivanov, B.N. (1998) Aust. J. Plant Physiol. 25, 673–678.
[3] Stemler, A. (1986) Biochim. Biophys. Acta 850, 97–107.
[4] Lu, Y.K. and Stemler, A.J. (2002) Plant Physiol. 128, 643–649.
[5] Pronina, N.A., Alakhverdiev, S.I., Kupriyanova, E.V., Klyachko-Gurvich, G.L. and Klimov, V.V. (2002) Fiziol. Rast. 49, 341–349.
[6] Moskvin, O.V., Razguliayeva, A.Y., Shutova, T.V., Khristin, M.S., Ivanov, B.N. and Klimov, V.V. (1999) in: Photosynthesis: Mechanism and Effects (Garab, G., Ed.), Vol. II, pp. 1201–1204, Kluver AP, Dordrecht.
[7] Moskvin, O.V., Shutova, T.V., Khristin, M.S., Ignatova, L.K., Villarejo, A., Samuelsson, G., Klimov, V.V. and Ivanov, B.N. (2004) Photosynth. Res. 79, 93–100.
[8] Villarejo, A., Shutova, T.V., Moskvin, O., Forssen, M., Klimov, V.V. and Samuelsson, G. (2002) EMBO J. 21, 1930–1938.
[9] Kukabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[10] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[11] Khristin, M.S., Nikitishena, O.V., Smolova, T.N. and Zastrizhnaya, O.M. (1997) Biol. Membr. 14, 133–142.
[12] Peter, G.F. and Thornber, J.P. (1991) J. Biol. Chem. 266, 16745–16754.
[13] Bassi, R.L., Rigoni, F. and Giacometti, G.M. (1990) Photochem. Photobiol. 52, 1187–1206.
[14] Lowry, J.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Winterman, J.F.G.M. and De Mots, A. (1965) Biochim. Biophys. Acta 109, 448–453.
[16] Ono, T. and Inoue, Y. (1983) FEBS Lett. 165, 255–260.
[17] Lindskog, S. and Malmström, B.G. (1962) J. Biol. Chem. 237, 1129–1137.
[18] Tripp, B.C., Smith, K. and Ferry, J.G. (2001) J. Biol. Chem. 276, 48615–48618.